NAVAL HEALTH RESEARCH CENTER

PCR ANALYSIS OF EGYPTIAN RESPIRATORY ADENOVIRUS ISOLATES, INCLUDING IDENTIFICATION OF SPECIES, SEROTYPES, AND COINFECTIONS

D. Metzgar
M. Osuna   *M. D. Saad*
S. Yingst   *A. Kajon*
M. Rakha   *J. Wu*
K. Earhart   *G. C. Gray*
D. Elyan   *M. A. K. Ryan*
H. Esmat   *K. L. Russell*

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PCR Analysis of Egyptian Respiratory Adenovirus Isolates, Including Identification of Species, Serotypes, and Coinfections

David Metzgar,[1]* Miguel Osuna,[1] Samuel Yingst,[2] Magda Rakha,[3] Kenneth Earhart,[2] Diaa Elyan,[2] Hala Esmat,[3] Magdi D. Saad,[2] Adriana Kajon,[4] Jianguo Wu,[1] Gregory C. Gray,[5] Margaret A. K. Ryan,[1] and Kevin L. Russell[1]

*Department of Defense Center for Deployment Health Research, Naval Health Research Center, San Diego, California[1];
U.S. Naval Medical Research Unit No. 3, Cairo, Egypt[2]; Ministry of Health, Arab Republic of Egypt, Cairo, Egypt[3];
Lovelace Respiratory Research Institute, Albuquerque, New Mexico[4]; and College of Public Health,
University of Iowa, Iowa City, Iowa[5]*

Eighty-eight adenovirus (Ad) isolates and associated clinical data were collected from walk-in patients with influenza-like illness in Egypt during routine influenza surveillance from 1999 through 2002. Respiratory Ad distributions are geographically variable, and serotype prevalence has not been previously characterized in this region. Serotype identity is clinically relevant because it predicts vaccine efficacy and correlates strongly with both clinical presentation and epidemiological pattern. Species and serotype identities were determined using several well-validated multiplex PCR protocols culled from the literature and supplemented with a few novel primer sets designed to identify rare types. The isolates included common species B1 serotypes (Ad3 and Ad7), common species C serotypes (Ad1, Ad2, and Ad5), the less common species B2 serotype Ad11, and three isolates of the rare species B1 serotype Ad16. Two isolates that appear to be variant Ad16 were also identified. Fifteen coinfections of multiple adenoviral types, primarily AdB/AdC and Ad3/Ad7 dual infections, were detected. The majority of these were verified using redundant PCR tests targeted at multiple genes. PCR is able to resolve coinfections, in contrast to traditional serum neutralization tests. PCR is also comparatively rapid and requires very little equipment. Application of the method allowed an inclusive determination of the serotypes found in the Egyptian respiratory sample set and demonstrated that coinfections are common and may play a previously unrecognized role in adenovirus pathogenesis, evolution, and epidemiology. In particular, coinfections may influence adenoviral evolution, as interserotypic recombination has been identified as a source of emerging strains.

Adenoviruses (Ads) are a diverse group of double-stranded DNA viruses responsible for a wide variety of human ailments (28). Ad are categorized by species (AdA, AdB1, AdB2, AdC, AdD, AdE, and AdF), further by serotype (Ad1 to Ad51), and even further by genome type. These distinctions correlate strongly with antigenicity, clinical presentation, and epidemiological character (30), and the same groups are similarly clustered on the basis of genetic homology (11). Under most circumstances, the clinically important Ad species are those associated with febrile respiratory disease (AdB1, AdC, and AdE) since these can cause widespread outbreaks with severe clinical presentations, including viral pneumonia and death (17, 20, 25, 29).

Ad3 and Ad7 (both species B1 serotypes) can cause large disseminated outbreaks that affect both adults and children and can cocirculate in a given geographic area (18, 29). These two serotypes are often responsible for the most severe respiratory symptomologies associated with adenoviral infection (12, 16). Outbreaks of Ad3 and Ad7 occur worldwide and have been documented in Japan (20), Korea (13), the United Kingdom (7), the United States (4, 9), South America (17), China (21), and elsewhere. AdB also includes the less common serotypes 11, 14, 16, 21, 34, and 35, all of which have been, at least occasionally, associated with respiratory disease (4, 9, 28).

Military-recruit populations in the United States regularly suffer from high rates of febrile respiratory disease caused by the sole AdE serotype, Ad4 (a serotype that is relatively rare in civilian populations), and AdB serotypes 3, 7, 14, and 21. In these populations, outbreaks can encompass the majority of the population (28, 29).

The AdC species includes serotypes 1, 2, 5, and 6. These serotypes are commonly associated with febrile respiratory illness in children and are noted to be endemic in certain regions (28), a pattern sometimes shared with Ad3 (9). AdC serotypes are also associated with a wide variety of illnesses in immunocompromised patients (1) and, though more rarely, in healthy adults (7, 31). Serosurveys suggest that virtually all people are exposed to these Ads during childhood (15, 26). They can be retained in an asymptomatic carrier state until at least young adulthood (10) and may be actively shed long after symptomatic infection (9). These patterns strongly suggest a reason for the endemic nature of these Ads and also point to the problems that may be associated with assigning causality based upon viral detection alone. One controlled study of 18,000 children and infants found that many healthy children in nonepidemic situations tested positive for AdC by culture, though sick children were significantly more likely to test positive (3).

Clearly, serotype information is medically relevant. Serotype correlates with the severity and symptomology of disease, as

* Corresponding author. Mailing address: Naval Health Research Center, P.O. Box 85122, San Diego, CA 92186-5122. Phone: (619) 553-9106. Fax: (619) 553-0935. E-mail: metzgar@nhrc.navy.mil.

well as with epidemiological characteristics (12, 28, 31). Knowledge of circulating serotypes can also predict the potential usefulness of available vaccines. The worst epidemics, often caused by newly emerging genome types (as defined by restriction enzyme analysis), may occur in areas where the causal serotype was rare in recent times (25).

Adenovirus serotype (and, indirectly, species) is traditionally determined through neutralization tests, in which antibodies raised against specific serotypes are used to suppress cytopathic effect in tissue culture assays. These tests are technically demanding, lengthy (2 weeks), and require expensive and difficult-to-obtain antiserum. They also require active viral culture and associated biosafety measures, which are not available in most clinical settings (1).

Not surprisingly, serotype correlates very well with sequence polymorphisms in the genes coding for the primary antigenic determinants, including both the hexon coat protein and the receptor-binding fiber protein. This correlation allowed the development and validation of several multiplex PCR assays (1, 34, 35) which together are capable of discriminating all of the common respiratory Ad serotypes. This method of testing is far less demanding than traditional neutralization and relies on commonly available and inexpensive equipment and reagents.

PCR is also capable of revealing coinfections of multiple Ad serotypes. This has been shown analytically with artificial mixtures of Ad types (1, 35) and in at least one clinical isolate containing a dual infection of two species C Ad (1). In contrast, neutralization, by nature of its design, can only reveal one dominant serotype. Our own recent work with PCR-based identification has shown that coinfections may be common in specific populations or environments (G. Vora and D. Metzgar, submitted for publication). The clinical implications of these coinfections are not yet clear, but the availability and increasing popularity of molecular tests capable of recognizing multiple coinfecting serotypes should permit elucidation of their impact.

In this study, a series of PCR tests was used to identify the serotypes of Ad associated with influenza-like illness (ILI) in Egypt, a country with previously poor documentation of circulating respiratory Ad strains. Where coinfections were apparent from multiple PCR signals, secondary PCRs targeted at different genetic loci were used to verify the initial results.

MATERIALS AND METHODS

Sample collection and processing. Throat swab samples ($n = 88$) were collected as part of routine influenza surveillance from outpatients with ILI at Monira General Hospital (Cairo), Kitchener General Hospital (Cairo), and Alexandria Fever Hospital (Alexandria), all in Egypt. Patients who voluntarily donated samples were anonymous but were assumed to be representative of the general civilian Egyptian population. Thirteen samples were collected in Cairo and 75 in Alexandria, during the period from January 1999 to June 2002. Patients were recruited if their symptoms fit the World Health Organization definition of ILI or if, in the judgment of the physician, the patient was suffering from an ILI. All but two had fevers exceeding 38°C. Cough, headache, muscle ache, runny nose, fatigue, sore throat, and chills were all common (>50%). Some patients (>30%) also experienced vomiting. Isolates were obtained from patients aged 0 to 55 years, with about two-thirds of the isolates coming from infants, children, and adolescents (<18 years). Samples were tested for influenza and Ad at the Naval Medical Research Unit 3 facility in Cairo, Egypt. All samples used in this study tested negative for influenza. Ad was initially identified by immunofluorescence after growth on MRC-5 or H292 tissue culture cell lines.

Infected tissue culture fluid (ITCF) was collected and frozen at Naval Medical Research Unit 3 and sent frozen on dry ice to the Naval Health Research Center (NHRC) for further testing. Two-hundred-microliter aliquots of ITCF samples were extracted using an Epicenter MasterPure complete DNA and RNA purification kit (Epicenter, Madison, Wis.) per the directions for saliva. Extracts were stored frozen at −20°C, and remaining ITCF was refrozen and stored at −80°C for further analysis. While the strains identified herein were tested as tissue culture amplified isolates, the same techniques are fully applicable to original patient specimens. The NHRC laboratory routinely uses PCR on original specimens as a means of identifying and serotyping Ads collected from military-recruit populations, and this method has been thoroughly tested and validated using culture and microneutralization as the gold standard. It should be noted that many coinfections, which are often quite biased in titer, may yield amplicons for only one strain in original samples. It is recommended that ITCF (grown virus) be diluted 1:100 in water before extraction and PCR with these methods to minimize the risks of cross-contamination and cross-reaction.

Most of the primers used here were taken from the existing literature, and these were exhaustively tested against neutralization results in previous papers. Primers from existing papers included the Ad universal primers (35), the primers for the split species-specific multiplex testing for species set B and E and set A, C, D, and F (35); the species B serotype-specific multiplex primers testing for Ad3, Ad7, and Ad21 (34); the species C serotype-specific multiplex primers testing for Ad1, Ad2, Ad5, and Ad6 (1); and the Ad4-specific primers (14). Novel primers were developed to distinguish rare members of species B not covered by the existing tests (the results of these were only accepted when confirmed by a second method; see Results and Discussion). For purposes of further discussion, the reactions using these primers will be termed "novel monoplex PCRs."

Secondary tests were also used to verify apparent coinfections. For apparent multispecies (AdB/AdC) coinfections, initially identified by the hexon-targeted species-specific PCR (35), verification was performed with another published species-specific primer set targeted at the fiber (27), using the AdB-specific and AdC-specific primer pairs for monoplex PCRs. For verification of Ad3/Ad7 coinfections, initially identified by the hexon-targeted Ad3/Ad7/Ad21 multiplex PCR (34), a new multiplex was developed for Ad3, Ad7, and Ad21 targeted to the fiber gene. This PCR is not precisely paired to the initial hexon multiplex because Ad7h (a currently common Ad7 genome type) carries a recombined Ad3 fiber that replaces the normal Ad7 fiber (19). This aspect of the test and the implications as related to the data are described in more detail in Results and Discussion. All primers used in this paper are shown in Table 1, and all cycling conditions are included in Table 2.

PCRs were carried out as shown in the flow chart (Fig. 1). The species-specific multiplex (35) was divided into two parts, testing separately for set B and E and set A, C, D, and F. All PCR tests were done for all controls and all samples to make sure there were no cross-reactions. Reactions were performed as follows.

Multiplex PCRs, including both the initial and the verifying species-specific PCRs, the initial and verifying Ad3/Ad7/Ad21 species B serotype-specific PCRs, and the AdC serotype-specific PCRs, were performed using a QIAGEN multiplex kit (QIAGEN, Valencia, Calif.). Reaction mixtures contained 0.5× Q-Solution, 1× Multiplex buffer, 0.2 μM concentration of each primer (Integrated DNA Technologies, Coralville, Iowa), and 2 μl of extracted sample in 25-μl aqueous reactions.

Reaction mixtures for monoplex PCRs contained 1× Q-Solution (QIAGEN), 1× PCR buffer (Promega, Madison, Wis.), 0.6 μM concentration of each primer (Integrated DNA Technologies), 0.8 mM concentration of each deoxynucleoside triphosphate (Promega), 3 mM concentration of $MgCl_2$ (Promega), 1.25 U of Taq polymerase (Promega), and 2 μl of extracted sample in 25-μl aqueous reactions.

Cycling conditions included a 10-min final extension at 72°C and a final hold at 4°C. In regards to other parameters, reactions were cycled as shown in Table 2.

All products were mixed 5:1 with loading dye (Sigma Aldrich, St. Louis, Mo.) and run for 90 min at 125 V on 1.5% agarose (Sigma Aldrich) gels with ethidium bromide (Sigma Aldrich). Standard 100-bp DNA ladders were used as a reference (New England Biolabs, Beverly, Mass.). "Product ladders" were also used for the multiplexes, generated by balanced combination of all Ad types targeted by the multiplex. Gels were visualized and recorded by photography on a UV light box.

Due to the occasional appearance of faint gray bands resulting from mispriming between closely related species B serotypes, products were recorded as positive when a band of "normal" intensity was observed in the expected size range. Normal intensity was defined as being within the range observed among expected bands amplified from positive-control strains (Fig. 2; see also Results and Discussion, "Methodology").

TABLE 1. Adenovirus species- and serotype-specific primers

Primer name	Primer sequence	Target	Source or reference
Primers for primary PCRs			
Ad1	TTCCCCATGGCICAYAACAC	Any hexon universal	35
Ad2	CCCTGGTAKCCRATRTTGTA		
AdA1	GCTGAAGAAMCWGAAGAAAATGA	AdA fiber	35
AdA2	CRTTTGGTCTAGGGTAAGCAC		
AdB1	TSTACCCYTATGAAGATGAAAGC	AdB fiber	35
AdB2	GGATAAGCTGTAGTRCTKGGCAT		
AdC1	TATTCAGCATCACCTCCTTTCC	AdC fiber	35
AdC2	AAGCTATGTGGTGGTGGGGC		
AdD1	GATGTCAAATTCCTGGTCCAC	AdD fiber	35
AdD2	TACCCGTGCTGGTGTAAAAATC		
AdE1	TCCCTACGATGCAGACAACG	AdE fiber	35
AdE2	AGTGCCATCTATGCTATCTCC		
AdF1	ACTTAATGCTGACACGGGCAC	AdF fiber	35
AdF2	TAATGTTTGTGTTACTCCGCTC		
Ad11-F	GAAGTTTCAGATGAAGAAAGTA	Ad11 hexon	This work
Ad11-R	AGGACTTAAGTTTGTTTTCTGC		
Ad14-F	AAATGCTAATCTTGGACAGCAGTC	Ad14 hexon	This work
Ad14-R	AGCCGTCCAGTGGAAAACAGTAGT		
Ad16-F	GCTGGCATTCTGGACACGGAGTTG	Ad16 E2A	This work
Ad16-R	CCTCATGAAGGCCGTATATCCAG		
Ad35-F	GATTTCAGCTGAAAACGAATCT	Ad35 hexon	This work
Ad35-R	GTCTGGTGTTTCCAAACCTACA		
AdCF	TGCTTGCGCTHAAAATGGGCA	AdC fiber (pairs with next 4)	1
Ad1R	CGAGTATAAGACGCCTATTTACA	Ad1 fiber	1
Ad2R	CGCTAAGAGCGCCGCTAGTA	Ad2 fiber	1
Ad5R	ATGCAAAGGAGCCCCGTAC	Ad5 fiber	1
Ad6R	CTTGCAGTCTTTATCTGAAGCA	Ad6 fiber	1
Adeno4.U3	CAAGGACTACCAGGCCGTCA	Ad4 hexon	14
Adeno4.L1	TTAGCATAGAGCATGTTCTGGC		
Ad3F	GGTAGAGATGCTGTTGCAGGA	Ad3 hexon	34
Ad3R	CCCATCCATTAGTGTCATCGGT		
Ad7F	GGAAAGACATTACTGCAGACA	Ad7 hexon	34
Ad7R	AATTTCAGGCGAAAAAGCGTCA		
Ad21F	GAAATTACAGACGGCGAAGCC	Ad21 hexon	34
Ad21R	AACCTGCTGGTTTTGCGGTTG		
Primers for PCRs verifying coinfections			
Ad3FibF[a]	GTGTTAATCCACTTACCACTGC	Ad3 fiber (also Ad7h)	This work
Ad3FibR[a]	GTTCCCGCATTAGGAAGGAC		
Ad7FibF[a]	GTCTTACAATAGATGACACCGAC	Ad7 fiber (except Ad7h)	This work
Ad7FibR[a]	GTGGAGTTTTTAGGGATGAAAGGC		
Ad21FibF[a]	GTTGGTGTATCAGACACTGTG	Ad21 fiber	This work
Ad21FibR[a]	GACTTCACTGGTTGCTGTAGAAG		
HsgB1	TCTATTCCCTACCTGGAT	AdB hexon	27
HsgB2	ACTCTTAACGGCAGTAG		
HsgC1	ACCTTTGACTCTTCTGT	AdC hexon	27
HsgC2	TCCTTGTATTTAGTATC		

[a] Primers Ad3FibF, Ad3FibR, Ad7FibF, Ad7FibR, Ad21FibF, and Ad21FibR may be used as a multiplex.

Primer design and PCR verification. Novel monoplex primer sets were designed to target serotype-specific sequences in the hexon gene of AdB serotypes that were not included in the existing multiplexes. Primer sequences were chosen using an alignment of all adenoviral hexon sequences available in GenBank (http://www.ncbi.nlm.nih.gov/Entrez/index.html). The general rarity of these se-

rotypes makes multiplexing unnecessary, since these tests need only be used when a sample tests negative by the multiplex for common serotypes.

In the case of the novel monoplex reactions, there were not enough samples of the targeted strains in the NHRC archive to allow a legitimate validation study, and therefore the specificities and sensitivities of the assays could not be properly

TABLE 2. Cycling conditions

PCR step	No. of cycles	Cycling conditions (time in min:s [temp in °C]) for primer[b]								
		Universal	Species	B serotypes[c]	C serotypes	Serotype 4	Serotypes 11, 14, 16, and 35	Species B	Species C	B serotypes[d]
Denaturation	1	5:00 (95)	15:00 (95)	5:00 (95)	5:00 (95)	5:00 (95)	5:00 (95)	15:00 (95)	15:00 (95)	15:00 (95)
Denaturation	35	1:00 (94)	0:30 (94)	1:00 (94)	1:00 (94)	0:40 (94)	1:00 (94)	0:40 (91)	0:30 (94)	0:30 (94)
Annealing	35	1:00 (52)	1:30 (52)	1:00 (56)	1:00 (47)	0:40 (53)	1:00 (53)[a]	0:30 (44)	1:30 (44)	1:30 (58)
Extension	35	1:30 (72)	2:00 (72)	1:00 (72)	2:00 (72)	1:30 (72)	1:30 (72)	0:40 (72)	1:30 (72)	1:00 (72)

[a] Ad14 required a 63°C annealing temperature for specificity.
[b] Sources and/or references for primers are as follows: for universal and species primers, reference 35; C serotype primers, reference 1; serotype 4 primers, reference 14; serotype 11, 14, 16, and 35 primers, this work; and species B and C primers, reference 27.
[c] Source for B serotype primers, reference 34.
[d] Source for B serotype primers, this work.

measured. When these tests yielded positive results, secondary tests were performed to verify the results. Positive results for Ad16 were cross-validated by the size of the AdB-specific band in the species-specific multiplex. This band size is unique among AdB and is known to distinguish Ad16 (35). Positive Ad11 results were verified by sequencing amplicons from the Ad11-specific PCRs and comparing them to the available sequences in GenBank by using the Basic Local Alignment Search Tool family of programs (http://www.ncbi.nih.gov/BLAST/).

Primers were chosen for the new Ad3/7/21 species B serotype-specific multiplex by using all existing fiber gene data available on GenBank for species B Ads. Sequences were chosen as targets on the basis of both complete conservation within the target group and maximum divergence between target and nontarget groups. Primers were chosen to be approximately 20 bp in length and have 50% GC content. Hairpins, repetitive sequences, and (especially) 3′ matches to internal (nonterminal) sequences of other primers in the same multiplex were avoided.

Controls. Control strains and sources are listed in Table 3. All positive controls were previously grown in the College of American Pathologists (CAP)-certified NHRC virology laboratory, and all were originally serotyped by the Centers for Disease Control and Prevention (CDC), the American Type Culture Collection (ATCC), or microneutralization (22) in the NHRC laboratory.

RESULTS AND DISCUSSION

Epidemiology. Results of species and serotype identifications by PCR are shown in Fig. 3. The results show a surprisingly high proportion of AdB1 infection relative to AdC, compared with general large-scale survey analyses (31). However, the high proportion of AdB1 in this population may simply
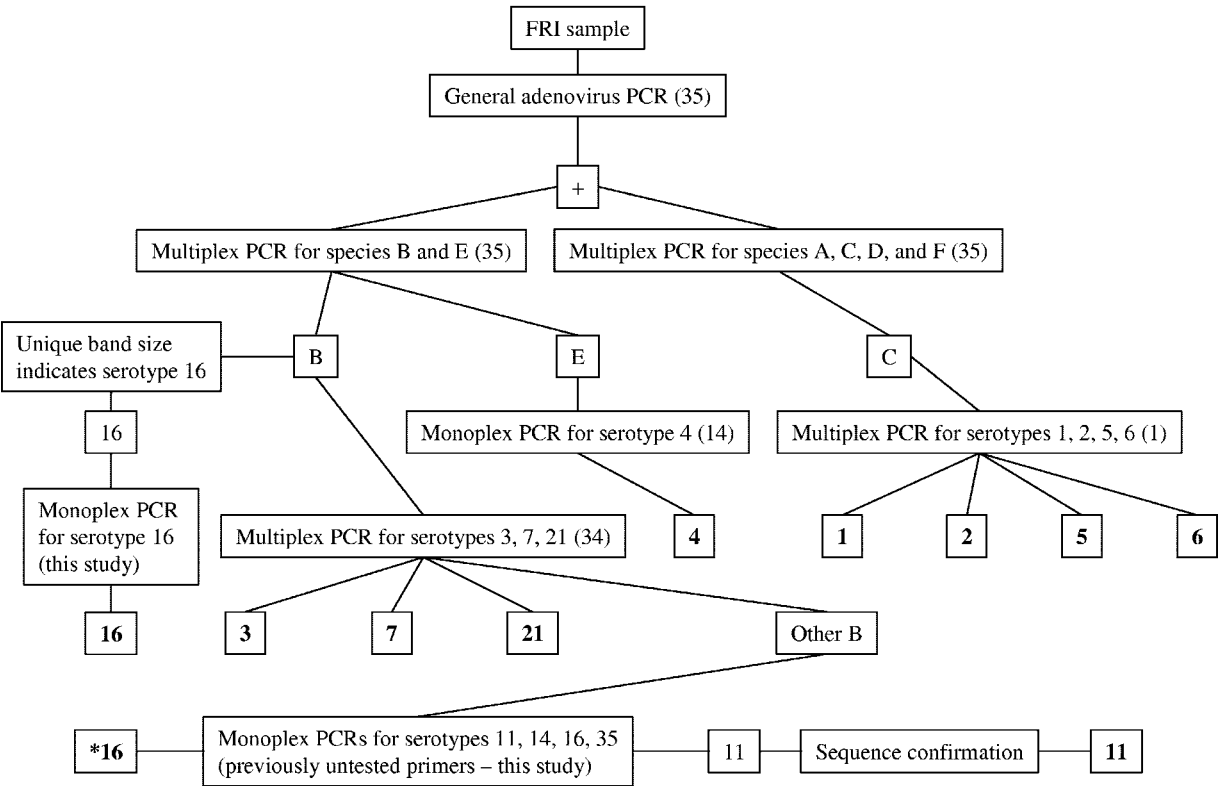

FIG. 1. Flow chart representing the series of PCRs used to identify strains in this study. FRI, febrile respiratory illness. Numbers in parentheses are reference numbers.
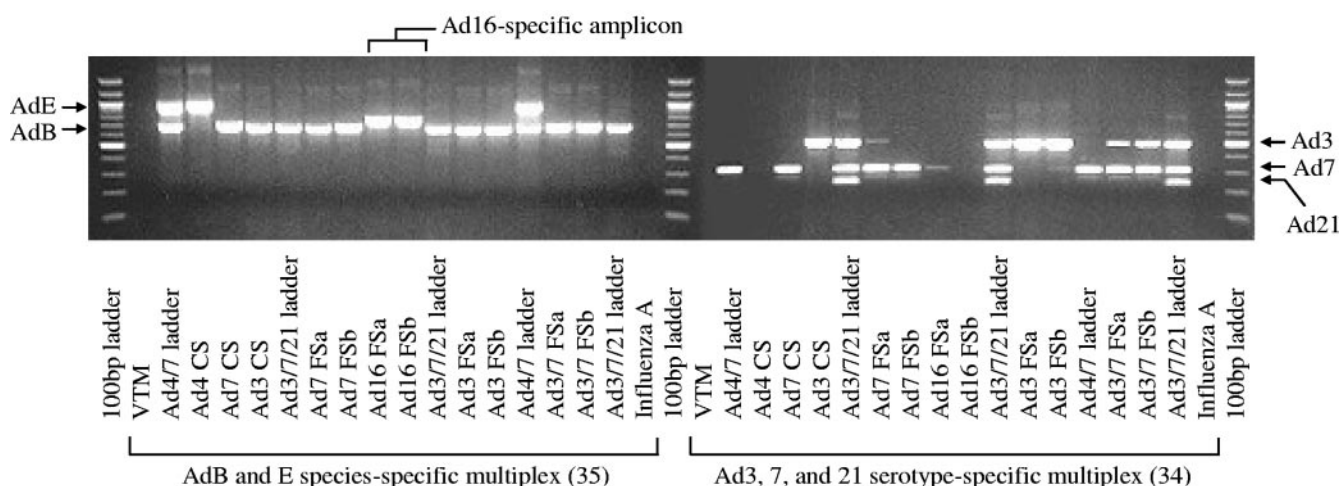
FIG. 2. Representative gels showing results with the AdB and AdE multiplex primers and the Ad3/Ad7/Ad21 multiplex primers for 20 samples. FS, field strain (isolate from Egypt); CS, control strain (previously serotyped isolate); VTM, viral transport medium (negative control). Ad4/7 ladder and Ad3/7/21 ladder are amplicons from control strain mixtures, termed product ladders, designed to enable easy band identification. Numbers in parentheses are reference numbers.

reflect a patient population suffering from relatively severe symptoms. Similar relative proportions of AdB and AdC were seen among hospitalized pneumonia patients in Korea (13).

Ad3 was by far the most common AdB serotype identified from patients with respiratory illness in Egypt, followed by Ad7. Four isolates of the less common Ad11 serotype were identified, as were three isolates of the rare Ad16 serotype and two apparent Ad16 variants (Ad16*). The two Ad16 variants could be subtyped within species B1 only by restriction enzyme analysis (A. Kajon, unpublished data), because they did not show the characteristic Ad16-specific band in the AdB/AdE multiplex (they yielded normal AdB amplicons). These appear to be unique strains and will be further analyzed. The Ad11 isolates tested positive with the novel Ad11 monoplex PCR and were firmly identified as Ad11 by sequencing of the amplicon from the Ad11 primer set.

For the most part, these proportions reflect those seen in other countries, including the United Kingdom (7), the United States (3, 9), Korea (13), and China (21), and in the world as a whole (31). As is the case here, Ad3 and Ad7 usually dominate AdB respiratory disease. The relative dominance of Ad3 and Ad7, the two serotypes implicated in the worst outbreaks (12), tends to vary both temporally and spatially, perhaps because of the protective effect of mass immunity imposed by recently circulating strains (25). The number of AdB serotypes other than Ad3 or Ad7 is considerably higher in this sample set than in other similar studies (7, 9), but the sample size is small enough that this could simply be chance.

The presence of Ad16 in the studied collection is noteworthy because this serotype is rarely reported in the literature in association with disease. Two of the three patients with typical Ad16 infections were infants (<1 year), one male and one female, while the other was a 30-year-old female. Clinical specimens from all three patients were collected in Alexandria but were spread over an 8-month period and do not appear to be epidemiologically related. Since this serotype is not the focus of specific surveillance and since neutralization tests are often not extended to theoretically rare serotypes, it is possible that this serotype is often underdiagnosed. Ad16 may also be uniquely important in this region. The prototype Ad16 (strain Ch 79) was previously isolated in the Middle East from a conjunctivitis/trachoma patient in Saudi Arabia (2, 23). Ad16 has occasionally been reported outside the Middle East by broad surveys, associated with symptomologies including respiratory disease (4, 33). In these cases, isolations of this virus were too rare (<1%) for strong measures of association with specific symptoms, and controlled surveys have found similar frequencies of isolation from ill patients and healthy controls (4). In contrast to this, one striking report from Arkansas reported two separate deaths, one adolescent and one adult, one from Reye's syndrome-like symptoms and the second a viral pneumonia, both associated with Ad16 infection in otherwise physically healthy patients (23). In these cases, the virus

TABLE 3. Control strains

Control strain[a]	Source	Identity by PCR
Ad12 (Huic)	CDC[b]	AdA
Ad3 (GB, VR-3)	ATCC	AdB, Ad3
Ad7 (Gomen)	ATCC[b]	AdB, Ad7
Ad1 (ws 2/5/91)	CDC[b]	AdC, Ad1
Ad2	CDC	AdC, Ad2
Ad5 (VR-1516)	ATCC	AdC, Ad5
Ad8 (ws 2/18/91)	CDC[b]	AdD
Ad4 (NIH 5/66)	CDC[b]	AdE, Ad4
Ad40 (Dugan)	CDC[b]	AdF
Influenza A	ATCC	None
Influenza B	ATCC	None
hMPV	NHRC	None
Healthy patient	NHRC	None
Water	Sigma	None
VTM (blank)	Remel	None

[a] Strain designations in parentheses are as listed by provider. hMPV, human metapneumovirus; VTM, viral transport medium (Remel, Lenexa, KS).
[b] These strains were generously provided by Dean Erdman and CDC. Other strains were obtained from CDC Biologicals or ATCC or were contained within archives from NHRC surveillance.
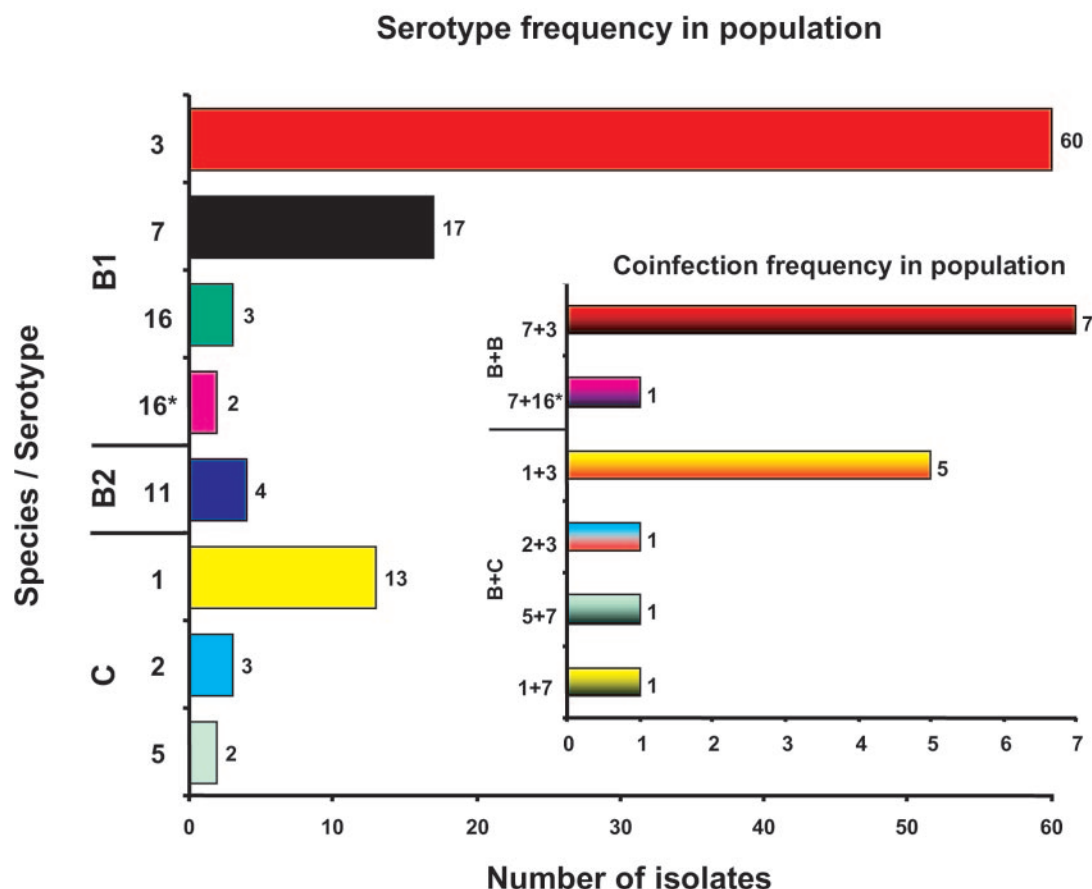
FIG. 3. Serotype and coinfection distributions of 88 adenovirus isolates from Egypt. Identified strains are grouped by species and further by serotype. All single-serotype counts include isolates from individuals with coinfections. Coinfection counts show the numbers of samples that tested positive for multiple serotypes. Colors in coinfection frequency bars represent the paired components of the coinfections. Ad16* and the coinfection of Ad7 and Ad16* refer to isolates that gave serotype-specific positive results for Ad16 by either serotype-specific PCR or restriction enzyme analysis but yielded only a normal AdB species amplicon with the species-specific primers. Since Ad16 is expected to yield a uniquely sized amplicon for this serotype (see Fig. 2 and Results and Discussion), these results represent ambiguous Ad16 positive results. These are either sequence variants of Ad16 or new serotypes most closely related to Ad16.

was isolated from parenteral organs related directly to the symptoms (spleen and lung, respectively). Given the rarity of isolation of Ad16 in general, these two deaths are a good reason to test for this serotype in cases of Ad-associated disease. If the two Ad16 variants (Ad16*) seen here are also considered, the proportion of Ad16 among respiratory disease-associated Ad isolates in this population is extremely high.

A similar situation is found in the case of Ad11. Ad11 is associated with acute hemorrhagic cystitis, both through naturally acquired infections (24) and, sometimes fatally, as the result of the virus hitchhiking with transplanted kidneys and bone marrow (6). It was also associated with fatal pneumonia in an immunocompromised patient (32). Like Ad16, Ad11 is rarely isolated in respiratory illness surveys (9) and strong causal correlations are generally lacking. However, serosurveys show that Ad11 is generally rare (8), so associations with specific symptomologies are suggestive of a causal etiology (33).

Certainly, future studies of respiratory Ads should not exclude these rare but potentially devastating serotypes methodologically, and it should be kept in mind that an understanding of the local distribution of Ads demands the use of maximally inclusive methods.

AdC serotypes, which are generally endemic among children, were also found. Identified serotypes included Ad1, Ad2, and Ad5. These are common, and their occurrence is expected given the age distribution of the patients from which they were isolated. It is important to remember that these, unlike species B1 Ads, usually establish long-lasting latent infections characterized by persistent intermittent excretion (3, 9). Therefore, positive results do not necessarily reflect a causal link between the viruses and recent symptoms.

The data collected during this study did not reveal any strong temporal or spatial trends within Egypt during the study period. Table 4 shows the distribution of serotypes for each year studied, while Table 5 shows the separate distributions of serotypes collected in Cairo and Alexandria.

This study revealed many Ad coinfections (samples containing multiple adenoviral types [see Fig. 2, 3, 4, and 5]). These were often identified from multiple bands arising in multiplex PCR tests (as exemplified in the right half of Fig. 2 by Ad3/7

TABLE 4. Serotype distribution by year of isolation

Yr of isolation	No. of isolates of serotype							
	Ad3	Ad7	Ad16	Ad16*	Ad11	Ad1	Ad2	Ad5
1999	4	3	0	0	0	2	0	1
2000	17	3	1	1	3	3	2	0
2001	34	9	2	1	0	7	1	1
2002	5	2	0	0	1	1	0	0
Total	60	17	3	2	4	13	3	2

TABLE 5. Serotype distribution by city of isolation

City of isolation	No. of isolates of serotype							
	Ad3	Ad7	Ad16	Ad16*	Ad11	Ad1	Ad2	Ad5
Cairo	10	5	0	0	0	4	0	1
Alexandria	50	12	3	2	4	9	3	1
Total	60	17	3	2	4	13	3	2

FSa and FSb). Many coinfections involved serotypes from both species AdB and species AdC, while others involved multiple serotypes of AdB.

All of the AdB/AdC coinfections were partially verified by secondary identification of two different serotypes corresponding to the two initially identified species. However, the serotype-specific tests and species-specific tests overlapped in terms of the targeted loci. The original species-specific test (35) and the species C serotype-specific test (1) both target the fiber gene, while the species B serotype-specific test (34) targets the hexon. Hence, this initial verification could not obviate the possibility of a single cross-reactive or recombined locus that might yield a double signal as an artifact in both tests.

This shortcoming was addressed through further verification of the coinfections by using redundant tests targeting independent loci. AdB/AdC coinfections originally identified using the fiber gene (35) were retested using secondary AdB- and AdC-specific primers targeting the hexon gene (27). Of eight initially identified AdB/AdC coinfections, seven were positive for both

AdB and AdC by the secondary test and were hence verified as coinfections (Fig. 4 shows examples of these data). Seventy-two of the 80 samples that initially appeared to be monoinfections of just one species also appeared to be monoinfections of the same species by the secondary test. The remaining eight samples that were initially identified as monoinfections (of AdB) were all verified as AdB but also yielded weak AdC-positive results by the second test (Ad7 FSa in Fig. 4 provides an example), suggesting that there may be more coinfections than originally recognized and also that AdC may often be found at a lower titer than AdB in AdB/AdC coinfections. Probability tree calculations addressing the overall level of correlation between the two coinfection assays yielded a P value of 0.000013 (representing the probability of both tests agreeing on 79 out of 88 samples by chance; data and calculations not shown). These results strongly validate the idea that the apparent coinfections are real and that they involve whole genomes as opposed to recombined parts or individually cross-reacting intermediate sequences.

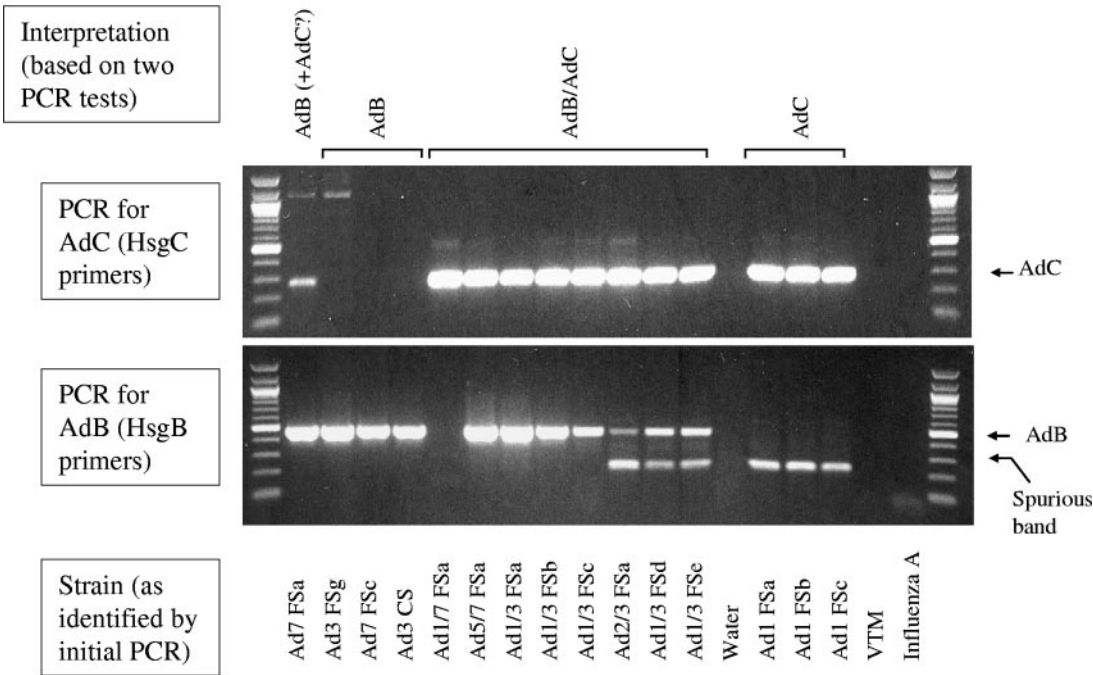Ad3/Ad7 coinfections presented a more complex problem in

FIG. 4. Representative PCR results for the secondary test (27) used to verify AdB/AdC coinfections. The gel includes amplicons (generated separately for AdB and AdC) from eight samples initially identified as AdB/AdC coinfections, three samples initially identified as AdB monoinfections, and three samples initially identified as AdC monoinfections, along with various controls. Initial identifications are indicated by the strain names. See the text for details.
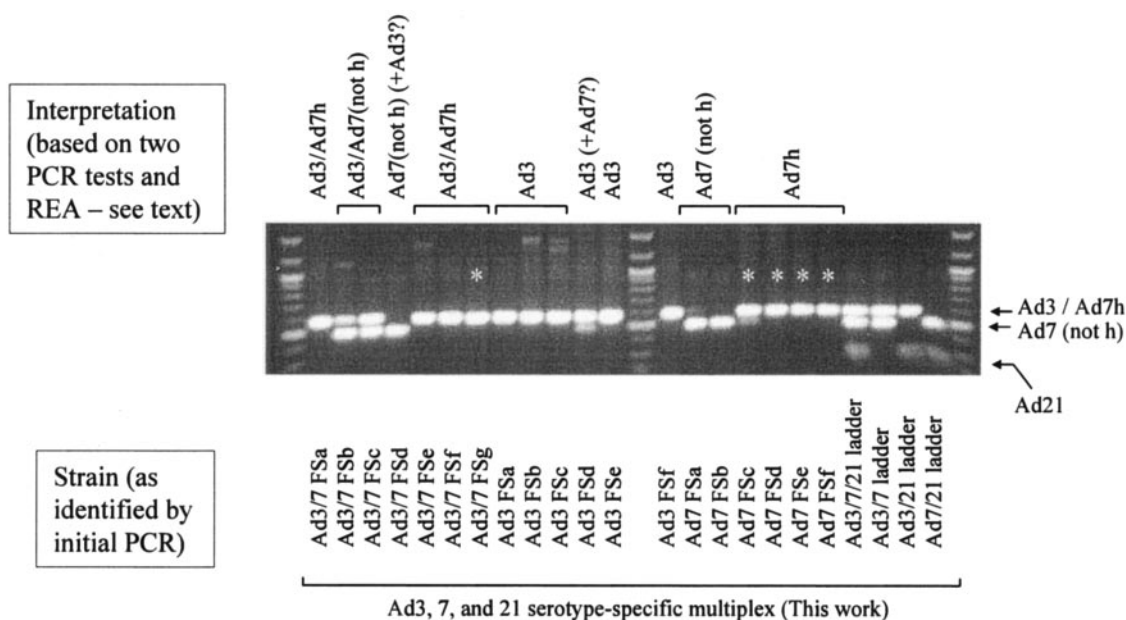
FIG. 5. PCR results for the secondary test used to verify Ad3/Ad7 coinfections (initially identified by the primary PCR [see Fig. 2]). The gel includes amplicons for all seven initially identified coinfections (identified as Ad3/7 FS), followed by six samples identified as Ad3 (Ad3 FS), six samples identified as Ad7 (Ad7 FS), and several control samples simulating coinfections of Ad3, Ad7, and Ad21. The PCR used here targets the fiber gene, which is identical in Ad3 and Ad7h (as contrasted with other Ad7 genome types). See the text for further discussion. *, Ad7h results that were definitively verified by whole-genome restriction enzyme analysis (REA). Note that coinfections are difficult to analyze by REA, so other Ad7 isolates, especially those in apparent coinfections, could also be Ad7h.

terms of verification because of the well-acknowledged circulation in nature of interserotypic recombinants (5, 19). The original results were obtained from the initial AdB serotype-specific multiplex (34) targeting the hexon. The fiber gene was the only other locus for which there was enough available sequence to develop an AdB serotype-discriminating PCR test. However, one of the most common currently circulating Ad7 genome types, Ad7h, carries a recombined Ad3 fiber that replaces the usually distinct Ad7 fiber (19). A new multiplex was designed for Ad3, Ad7, and Ad21 based on fiber sequences, with the understanding that this would really test for the Ad21 group versus the Ad7 group (except Ad7h) versus the Ad3-Ad7h group and hence would identify coinfections of Ad3 and Ad7h as Ad3 only (or rather, as Ad3-Ad7h only). This multiplex was validated with a set of Ad3, Ad7, and Ad21 isolates previously identified by antiserum neutralization in the NHRC virology laboratory, using both individual controls and mixed (artificial-coinfection) controls (most of these data are not shown, though the artificial-coinfection control mixtures are included in the sample set pictured in Fig. 5). The new multiplex was then used to test the apparent Ad3/Ad7 coinfections.

The results of this test are shown in Fig. 4, with results for the seven apparent Ad3/Ad7 coinfections, six apparent Ad3 monoinfections, and six apparent Ad7 monoinfections. Two of the initially apparent Ad3/Ad7 coinfections were verified by the new method, while one yielded only an Ad7(not h) band and four yielded only an Ad3-Ad7h band (Fig. 5). These last four results are still consistent with Ad3/Ad7h coinfection but only if approximately two-thirds of Ad7 in Egypt is in fact Ad7h. This possibility is supported by the results from the initially apparent monoinfections. While all of the purported

Ad3 monoinfections yielded an Ad3-Ad7h band, four of the purported Ad7 monoinfections yielded an Ad3-Ad7h band and only two yielded an Ad7(not h) band. Furthermore, whole-genome restriction enzyme analysis (genome typing) supported the contention that the four samples that were Ad7 by hexon and Ad3-Ad7h by fiber were in fact genome type Ad7h (Kahon, unpublished). The most consistent interpretation of all of the results is shown in Fig. 5.

The general absence of reported coinfections in the literature may well be the result of methodological limitations. Neutralization tests are comparative and qualitative and are inherently biased towards identification of a single serotype. Multiplex PCR, on the other hand, can amplify minority targets as effectively as majority targets and is biased in the other direction, towards identification of serotypes even in cases where one coinfecting serotype is present at a much lower titer than the other.

Recombination, which requires coinfection, appears to play a role in Ad evolution (19). The clearest example of this is Ad7h, which appears to be Ad7p with a recombinationally replaced Ad3p fiber gene (19). Various subtypes of Ad7h may also be derived from further recombinations of Ad7 and Ad3 (5). Since the most common coinfection seen in this sample set is Ad7/Ad3, the potential evolutionary implications of coinfections are clear.

It can be seen by comparison of the monoinfection and coinfection charts (Fig. 3) that the common coinfections tend to involve the most common serotypes. This suggests that the composition of coinfections may be determined by chance associations of multiple serotypes rather than by unique features of specific pairings. The clinical significance of adenoviral coin-

fections, like the significance of Ad11 and Ad16, can only be understood through further surveillance and analysis.

Methodology. PCR provides a rapid and inclusive method of serotype identification. Of 88 strains, the method successfully identified a species in all cases and a serotype in 86 cases. The two remaining untypeable isolates have now been studied further through restriction enzyme analysis and appear to be variants of Ad16 (Ad16*) (Kahon, unpublished). In all cases except one (a possible coinfection identified as Ad7 plus Ad16*), positive serotype identifications were supported by species results, including cases for which multiple serotypes (coinfections) were observed.

The ability of PCR to identify multiple coinfecting Ad types was strongly supported by the results. Coinfections were identified at both species and serotype levels, and multiple tests designed to cross-validate apparent coinfection signals by using separate genetic loci as targets correlated well with one another.

Results in general were clear and conclusive. All control strains and negative controls yielded results consistent with expectations, strengthening the already intensive validations of the literature-derived multiplex PCRs used here. The novel monoplex primers developed and used to determine the serotypes of otherwise unidentifiable isolates also served their purpose. Identifications of Ad11 were easily verified with sequencing, which is available to any PCR-enabled laboratory at low cost through commercial sequencing services. Identifications of three of the Ad16 isolates found here were readily verified with reference to Ad16-specific signatures from the species-specific (AdB and AdE) multiplex. The other two apparent Ad16s (Ad16*) matched some known Ad16 molecular signatures but not others. This may well be because so little molecular data exist for Ad16 and hence the range of molecular signatures found among Ad16 is not yet well defined. The relative ease of PCR test design and implementation allowed all of the infections to be diagnosed with enough resolution to reveal the impact of potentially underestimated serotypes.

The AdB serotype-specific test (34) relies on rare polymorphisms in the hexon gene to distinguish these very closely related serotypes. It was found that the primers used in this test are capable of producing weak (gray) "shadow bands" for closely related serotypes when used to test some high-titer ITCF isolates. This can be seen in two of the cases shown in the Ad3, 7, and 21 portion of Fig. 2 (Ad7 FSb and Ad16 FSa). The NHRC laboratory recently saw similar bands with control samples used in other studies. In both of the cases noted in Fig. 2, independent monoplex tests for the serotypes suggested by the shadow bands gave negative results. Therefore, the data collected in this study were analyzed under a criterion for positive results that demands a clear band, within the intensity range seen among correct bands for positive-control samples. Further efforts toward clinical validation of these methods suggested that the observed shadow bands can be avoided by 10- or 100-fold dilution of ITCF samples prior to extraction. The high-titer results are shown here for illustration of the potentially ambiguous results. In any case, these shadow bands are readily distinguishable from the much brighter bands resulting from specific amplification.

The inclusiveness of this method, extending to both rare serotypes and coinfections of multiple serotypes, makes it an attractive and accessible alternative to classic culture and neutralization methods. This method serves to identify not only serotype but also presence/absence and species and in that sense offers a complete method of analysis that can be performed on either grown virus or original patient specimens.

Application of the PCR typing method to a unique and diverse sample set from Egypt allowed nearly complete description of the species and serotype identities of the collected adenovirus strains. Furthermore, the strengths of such molecular analyses in identifying coinfections became clear, offering a powerful tool for investigating the dynamics of genetic evolution and recombination events in this important human pathogen.

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